An indirect enzyme linked immunosorbent assay for the detection of bovine antibodies to multiple *Leptospira* serovars

Om Surujballi, Maria Mallory

Abstract

An indirect enzyme linked immunosorbent assay was developed for the detection of bovine antibodies to multiple pathogenic *Leptospira* serovars, including *canicola*, *copenhageni* (represents *icterohaemorrhagiae*), *grippotyphosa*, *hardjobovis*, *pomona*, and *sejroe*. The antigen utilized in this assay was a sonicated mixture of equal parts of killed whole cells of each of the 6 serovars named above. A mouse monoclonal antibody against bovine immunoglobulin (Ig)G_1 that was conjugated with horseradish peroxidase was used for detection of bound antibodies. This assay was evaluated with sera (n = 3107) that were microscopic agglutination test (MAT)-negative (at a 1:100 dilution) for each of the 6 serovars listed above and sera (n = 601) that were MAT-positive (at a 1:100 dilution) for 1, or any combination of the 6 listed serovars. In addition, sera from serial weekly bleedings of cows, which were individually experimentally infected with serovars *hardjobovis*, *copenhageni*, *grippotyphosa*, or *canicola*, were also tested in this assay.

At an optimal cut-off point determined by receiver operating characteristic (ROC) curve analysis, the relative sensitivity and specificity of the assay were 93.5% (95% confidence interval = 91.2% to 95.3%) and 94.7% (95% confidence interval = 93.9% to 95.5%), respectively. This assay was able to detect antibody in the sera of animals experimentally infected with serovar *hardjobovis* as early as 1 week postinoculation

Résumé

Une épreuve ELISA indirecte a été développée afin de détecter chez les bovins des anticorps envers des sérovars pathogènes de Leptospira, *tels* canicola, copenhageni *(représentant* icterohaemorragiae*),* grippotyphosa, hardjobovis, pomona *et* serjoe. *L'antigène utilisé dans cette épreuve était un mélange soniqué de quantités égales de cellules entières tuées de chacun des 6 sérotypes mentionnés. Un anticorps monoclonal de souris dirigé contre les immunoglobulines bovines de type IgG1 et conjugé à la peroxydase de raifort a été utilisé pour la détection des anticorps liés. Cette épreuve a été évaluée avec des sérums (*n = *3107) qui donnaient un résultat négatif lors de l'épreuve d'agglutination microscopique (MAT) (à une dilution de 1:100) pour chacun des 6 sérovars mentionnés précédemment et des sérums (*n = *601) qui étaient MAT-positifs (à une dilution de 1:100) pour 1, ou toute combinaison des 6 sérovars listés. De plus, des échantillons de sérum provenant de vaches infectées expérimentalement de façon individuelle avec les sérovars* hardjobovis, copenhageni, grippotyphosa *ou* canicola *ont été prélevés sur une base hebdomadaire pendant plusieurs semaines et ont été testés avec cette épreuve.*

Au seuil optimal déterminé par la courbe d'analyse, la sensibilité et la spécificité relatives de l'épreuve étaient respectivement de 93,5 % (intervalle de confiance de 95 % = 91,2 % à 95,3 %) et 94,7% (intervalle de confiance de 95 % = 93,9 % à 95,5 %). Cette épreuve permettait de détecter les anticorps sériques d'animaux infectés expérimentalement avec le sérovar hardjobovis *dès la 1ère semaine post-inoculation.*

(Traduit par Docteur Serge Messier)

Introduction

Leptospirosis is a zoonotic disease which affects a wide range of economically important livestock. The causative organisms belong to the genus *Leptospira*, which contains over 200 serovars (1). In any given geographic location, only relatively small numbers of serovars are endemic, and these tend to be maintained in specific animal host species (2). In cattle, this disease causes agalactia, abortions, stillbirths, the birth of weak calves, and possible infertility on a worldwide basis (2).

In Canada, the *Leptospira* serovars associated with cattle are predominantly *hardjo* (now generally recognized as *hardjobovis*) and *pomona*, and to lesser extents *grippotyphosa* and *icterohaemorrhagiae* (3–10). Currently in Canada, there is a requirement to monitor cattle that are resident in artificial insemination (AI) centres for serovars *canicola*, *copenhageni* (represents *icterohaemorrhagiae*), *grippotyphosa*, *hardjobovis*, *pomona*, and *sejroe*. In addition, for the international trade of live cattle, semen, and embryos, many countries request testing for specific *Leptospira* serovars, which may include those listed above.

Animal Diseases Research Institute, Canadian Food Inspection Agency, 3851 Fallowfield Road, P.O. Box 11300, Station H, Nepean, Ontario K2H 8P9.

Address all correspondence and reprint requests to Dr. Surujballi; telephone: (613) 228-6698; fax: (613) 228-6670; e-mail: surujballio@inspection.gc.ca

Received November 12, 2002. Accepted July 14, 2003

© Minister of Public Works and Government Services Canada 2004.

The most commonly used and internationally accepted serological test for leptospirosis is the microscopic agglutination test (MAT) (11). However, the MAT has a number of serious problems which indicate the need to develop and implement alternative methods for diagnosing this disease.

This study reports the development and evaluation of an indirect enzyme immunoassay capable of detecting bovine antibodies to the 6 serovars of pathogenic *Leptospira* that are routinely monitored in Canada.

Materials and methods

Culture

Leptospira borgpetersenii serovars *hardjobovis* (field strain) and *sejroe* (strain M84); *Leptospira interrogans* serovars *canicola* (strain Hond Utrecht IV), *copenhageni* (strain M20), and *pomona* (strain Pomona); and *Leptospira kirschneri* serovar *grippotyphosa* (strain Moskva V) were grown at 29°C in SPL 5× *Leptospira* medium (Scientific Protein Laboratories, Waunakee, Wisconsin, USA) that was reconstituted according to the manufacturer's directions. Cell counts were determined with a Petroff-Hausser bacteria counter (Canadawide Scientific, Ottawa, Ontario).

The microscopic agglutination test

The MAT was performed in microtitre plates as described (11). Live 4-d *Leptospira* cultures with concentrations adjusted to McFarland Standard #0.5, were used as the antigens. The sera were diluted (serial 2-fold with a final volume of 50 μL) in phosphate buffered saline (0.01 M sodium phosphate, 0.145 M sodium chloride, pH 7.2 [PBS]), after which the antigens (50 μL) were added. The plates were incubated at room temperature for 1.5 h and then examined by darkfield microscopy. The MAT titre was the reciprocal of the highest dilution of the serum in which ≥ 50% of the antigen was agglutinated.

Field sera

Bovine field sera, which were submitted to Canadian Food Inspection Agency (CFIA) regional laboratories (Lethbridge, Alberta; Saskatoon, Saskatchewan; Nepean, Ontario; St. Hyacinthe, Quebec; Sackville, New Brunswick) for testing for antibodies against various organisms, were used in this study. The sera were collected from cattle of various ages and breeds on farms located in each of the 10 Canadian provinces. The sera collected outside of Ontario were shipped overnight to the Nepean laboratory. All sera were tested with the MAT for serovars *canicola*, *copenhageni*, *grippotyphosa*, *hardjobovis*, *pomona*, and *sejroe* prior to storage at −20°C. From this collection, panels consisting of 3107 sera, which were negative in the MAT (1:100 dilution) for each of these 6 serovars and 601 sera which were positive in the MAT (1:100 dilution) for at least 1 of the 6 serovars listed, were assembled and tested using the enzyme linked immunosorbent assay (ELISA).

Sera from experimentally infected cattle

Five heifers (approximately 18 mo old) were experimentally infected with serovars *canicola* ($n = 1$), *copenhageni* ($n = 1$), *grippotyphosa* ($n = 1$), and *hardjobovis* ($n = 2$) as part of another study (unpublished). One millilitre of a live 7-d culture (approximately 10^6 cells/mL) was instilled into each eye and nostril of each heifer (1 serovar per animal) on 3 consecutive days. The animals were bled 10 d after the initial inoculation and weekly thereafter. The sera obtained from these weekly bleedings were tested with the ELISA and MAT. All of the animals were housed and monitored according to the guidelines of the Canadian Council on Animal Care (12).

The ELISA antigen

Cells from 7-d cultures of each of the 6 serovars listed above were harvested by centrifugation (20 000 × *g*, for 30 min at 4°C) and washed twice by centrifugation (as above) in PBS. The cells were resuspended in PBS, counted, and the concentration adjusted to approximately 1×10^{10} cells/mL. The cells were killed by heating at 56°C overnight and then equal volumes of the cell suspension from each of the 6 serovars were combined. Aliquots (1.2 mL) of the mixture were then cooled on ice and sonicated (on-off cycle) for 2 min with a 375 W cell disruptor (Heat Systems-Ultrasonics, Farmingdale, New York, USA). The resulting material was stored at −20°C and used as the antigen in the ELISA.

The ELISA

The ELISA was performed in Nunc 2-69620 microtitre plates (Life Technologies, Burlington, Ontario). Unless specified, the plates were sealed with acetate covers and incubated for 1 h at room temperature for all stages of the assay. The plates were washed (4×) between reaction stages with PBS supplemented with 0.05% Tween 20 (PBST) in a microplate washer (Titertek microplate washer; Flow Laboratories, Mississauga, Ontario). All reagents were added at a volume of 200 μL/well. The antigen was diluted in 0.06 M sodium carbonate-bicarbonate buffer, pH 9.6. The sera and conjugate (a mouse monoclonal anti-bovine immunoglobulin [Ig]G_1 [designated M23] which was produced and conjugated to horseradish peroxidase in-house), were diluted in PBST. Optimal working dilutions of the antigen and conjugate were established by titration, and the serum was diluted 1:100. The substrate/chromogen solution consisted of 0.015% hydrogen peroxide and 1.0 mM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma Chemical Company, St. Louis, Missouri, USA) in 0.05 M sodium citrate buffer (pH 4.5). After addition of the substrate/chromogen solution, the plates were left uncovered and were continuously agitated on a plate shaker (Titertek plate shaker; Flow Laboratories). All plates were read at 10 min with a plate reader (Titertek Multiskan MCC plate reader; Flow Laboratories) equipped with a 414 nm filter.

Microtitre plates were coated with the antigen, incubated overnight at room temperature and then frozen at −20°C. Prior to use, the plate was thawed at room temperature and washed. The serum samples (each in a single well) were added and the plate incubated and washed. The conjugate was added next and the plate incubated and washed. Finally, the substrate/chromogen solution was added and the plates were read.

Six controls (each in duplicate wells) were included in every plate. Control number 1 (high positive control) consisted of a high titre serovar *hardjobovis* bovine antiserum. Controls 2, 3, and 4 were intermediate titre bovine antisera to serovars *pomona*, *sejroe*, and

copenhageni, respectively. Control 5 was a negative serum and in control 6, the diluent buffer (PBST) was used in place of the serum. The reagent parameters were adjusted so that the high positive control yielded an optical density $(OD)_{414}$ value of approximately 1.0 at 10 min of substrate/chromogen development.

Acceptance criteria and data expression

Two criteria were established to determine the acceptability of the results of this assay. The first was, that for any plate, the mean of the duplicate OD_{414} values of the high positive serum control must fall within upper and lower limits (mean $OD_{414} \pm 2$ standard deviations [*s*], respectively, of 25 tests). If this criterion was fulfilled, then the mean OD_{414} value of each of the 6 duplicate controls was expressed as a percentage of the mean OD_{414} value of the high positive control (% HPC). This was calculated according to the equation below.

$$\% \text{ HPC} = \frac{\text{mean OD}_{414} \text{ of control (or OD}_{414} \text{ of test sample)} \times 100}{\text{mean OD}_{414} \text{ of high positive control serum}}$$

The 2nd criterion used to determine acceptability of the results was, that for any plate, the mean % HPC value of only 1 of the controls was allowed to fall outside of the upper and lower limits (mean % HPC of at least 25 tests ± 2 *s*, respectively, for each control). If the results for the controls on a plate met both of these criteria, then the results for the test sera included on that plate were considered valid.

Data analysis

The frequency distributions of the % HPC values obtained for the positive and negative sera were determined. Receiver operating characteristic (ROC) curve analysis was then performed using a computer program (MedCalc Software, Mariakerke, Belgium). From the ROC curve analysis, paired estimates of sensitivity and specificity at various % HPC cut-off values were determined. The % HPC value that resulted in the highest sum of the sensitivity and specificity estimates was established as the cut-off point. The ROC curve was then plotted and the area under the curve (AUC) value calculated as a measure of the accuracy of the test.

Results

Field sera

The frequency distributions of the % HPC values obtained from testing the MAT-positive and the MAT-negative field sera are presented in Figures 1A and 1B, respectively. These histograms show that the majority of the positive sera resulted in % HPC values of > 40 and the majority of the negative sera yielded % HPC values of < 40. This indicated that the cut-off point for distinguishing between negative and positive results would be approximately 40% HPC. The ROC curve analysis of the ELISA data produced paired estimates of relative sensitivity and relative specificity at different cut-off points, selected examples of which are shown in Table I. A cut-off point of $\geq$ 42% HPC was recommended. At this cut-off point, the relative sensitivity and specificity estimates were 93.5% (95% confidence interval = 91.2% to 95.3%) and 94.7% (95% confidence interval = 93.9% to 95.5%), respectively. The ROC curve (Figure 2) had an AUC value of 0.977 (95% confidence interval = 0.972 to 0.982), which indicates a high level of accuracy for this ELISA.

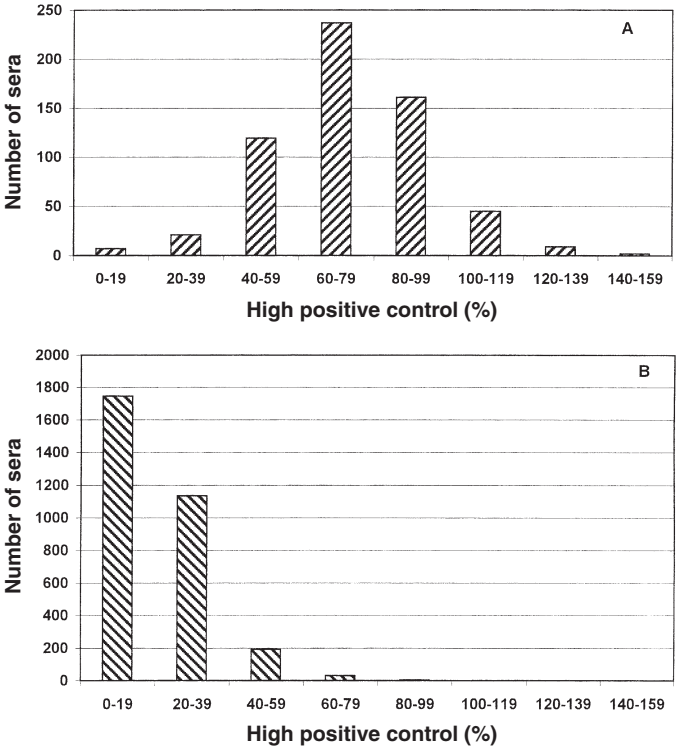

Figure 1. Frequency distribution of enzyme linked immunosorbent assay (ELISA) results of (A) 601 microscopic agglutination test (MAT)-positive field sera and (B) 3107 MAT-negative field sera. The x-axis shows high positive control (%) values and the y-axis shows the number of sera.

Table I. Effect of varying the cut-off value (selected examples shown) on the sensitivity and specificity of the enzyme linked immunosorbent assay (ELISA) as determined by receiver operating characteristic (ROC) curve analysis

Cut-off value (% HPC)[a]	% Sensitivity (95% CI)[b]	% Specificity (95% CI)
$\geq$ 12	99.7 (98.9–99.9)	28.8 (27.2–30.4)
$\geq$ 22	98.5 (97.2–99.3)	66.1 (64.4–67.8)
$\geq$ 32	96.3 (94.5–97.7)	86.8 (85.6–88.0)
$\geq$ 42[c]	93.5 (91.2–95.3)[c]	94.7 (93.9–95.5)[c]
$\geq$ 52	84.7 (81.6–87.5)	97.8 (97.2–98.3)
$\geq$ 62	70.4 (66.6–74.0)	99.2 (98.9–99.5)

[a] Percent high positive control
[b] Ninety-five percent confidence interval
[c] Optimal cut-off value

Sera from experimentally infected animals

This ELISA detected antibodies in the sera of each of the 2 cows that were experimentally infected with serovar *hardjobovis* as early as 1 wk postinoculation (Figure 3). The sera from the cows inoculated with serovars *canicola*, *copenhageni*, and *grippotyphosa* were ELISA-positive at wk 2, 3, and 4, respectively.

The MAT results of these sera were, with the exception of those from the cow inoculated with serovar *grippotyphosa*, very similar to the results obtained in the ELISA. The cows inoculated with serovars

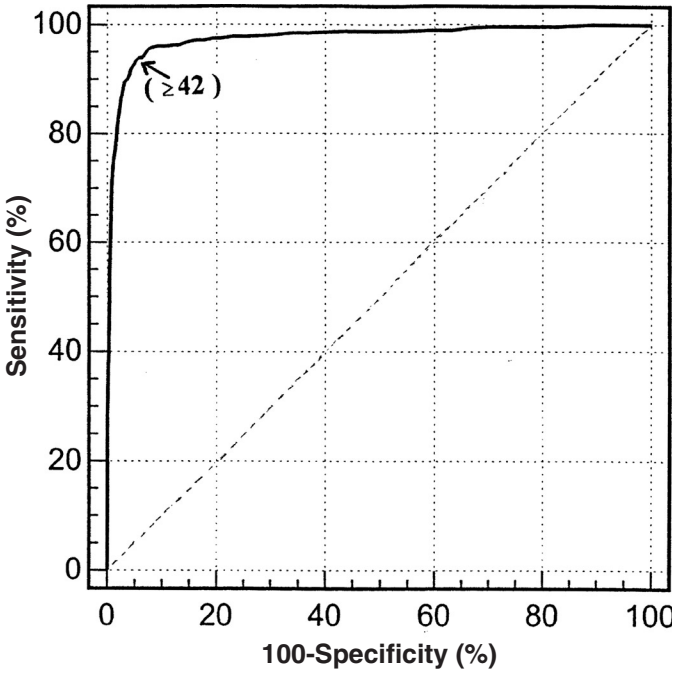

Figure 2. Receiver operating characteristic (ROC) curve obtained from the analysis of the enzyme linked immunosorbent assay (ELISA) results of 601 microscopic agglutination test (MAT)-positive and 3107 MAT-negative field sera. The false-positive rate (100-specificity [x-axis]) is plotted against the true-positive rate (sensitivity [y-axis]) for each high positive control (HPC) (%) cut-off point applied. An optimal cut-off point of ≥ 42% HPC is indicated (arrow). The area under the ROC curve = 0.977.

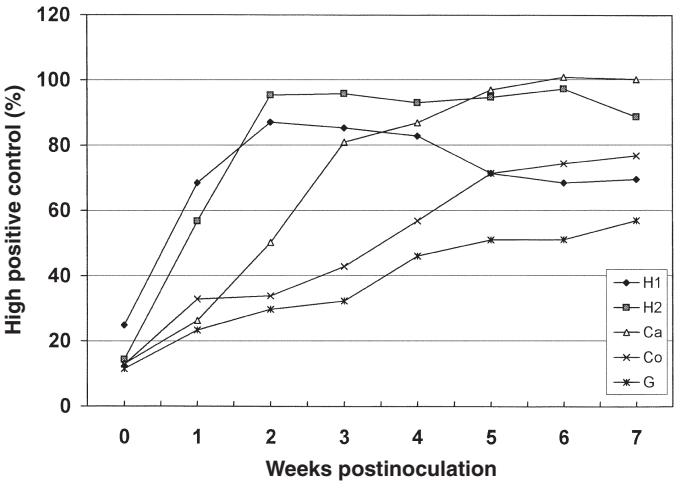

Figure 3. Enzyme linked immunosorbent assay (ELISA) results of sera obtained from serial bleedings of cattle experimentally infected with *hardjobovis* (H1 and H2), *canicola* (Ca), *copenhageni* (Co), and *grippotyphosa* (G). The x-axis shows time (weeks post-inoculation) and the y-axis shows percent high positive control (HPC) value. A cut-off value of ≥ 42% HPC is used to distinguish between positive and negative results.

hardjobovis, *canicola*, and *copenhageni* were MAT positive at wk 1, 2, and 3, respectively, (same as in the ELISA). The cow inoculated with serovar *grippotyphosa* was MAT positive at wk 3 (1 wk earlier than in the ELISA).

The ELISA quality control

The mean % HPC values for 4 of the 6 controls (the *copenhageni*-positive serum, the *pomona*-positive serum, the negative serum, and the diluent buffer), that were obtained from 51 assays are presented in Figure 4. The upper and lower control limits (mean % HPC ± 2 *s*, respectively) for each control are also indicated. Data are not presented for the *sejroe* control for reasons of clarity, since these overlap with the % HPC values that were obtained for the *copenhageni* control. Data are also not presented for the *hardjobovis* (high positive) control since it is arbitrarily set at 100% HPC for each plate. For each of the plates represented, the mean OD_{414} value for the duplicate *hardjobovis* control wells was within the established control limits, that is, the first criterion for accepting the results for that plate was fulfilled (data not shown). Figure 4 shows that for most of the assays, the mean % HPC values for each of the controls fell within the prescribed limits. The negative serum control and the *copenhageni* serum control values fell outside of the limits on 1 and 3 occasions, respectively. The *pomona* control fell outside of the limits 4 times and the diluent buffer control was always within the set limits. However, for these 51 plates, the results were accepted since only a single control value was outside of the prescribed limits on any given plate, that is, both of the quality control criteria were fulfilled.

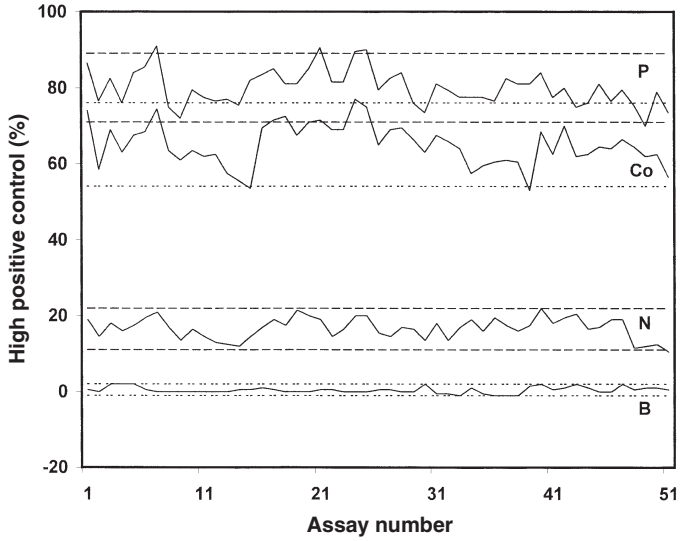

Figure 4. Quality assurance data obtained for the enzyme linked immunosorbent assay (ELISA). The y-axis shows the mean high positive control (%) values obtained for 4 (P = *pomona* serum, Co = *copenhageni* serum, N = microscopic agglutination test [MAT]-negative serum, and B = buffer) of the 6 quality controls reagents that are included in every ELISA plate. Upper and lower limits (± 2 *s*, respectively) for each control are indicated. The x-axis shows assay number of the 51 plates included in this analysis.

Discussion

Culture of an organism from a diseased host is probably the most definitive method of demonstrating infection. In the case of *Leptospira* however, there is a great deal of subjectivity and unreliability associated with culture. Furthermore, the need for rapid turn-around times for results has further precluded culture as a routine diagnostic tool for leptospirosis. As a result, diagnosis of this disease is mainly performed with serological tests. Of this genre, the MAT is the most widely used and accepted. However, the MAT has many disadvantages which indicate the need for an alternative test for routine diagnosis of leptospirosis. One major problem with the MAT

is its use of live organisms as antigens. This requires the continuous culture and handling of these hazardous bacteria in specialized laboratories. In addition, since it is usually required to demonstrate the absence of antibodies to more than 1 specific serovar, a serum sample is usually tested against multiple antigens to establish a negative status. The use of live organisms, the origin and handling of which may not be standard among laboratories, and the subjective assessment of results can also make quality assurance of the MAT difficult. Another problem associated with the MAT is that it only detects agglutinating antibodies and, as such, non-agglutinating antibodies may go undetected. This may account for some reports of *Leptospira* being cultured from animals without the presence of any detectable MAT antibody titre (7,13–18). As a consequence of these problems associated with the MAT, we are developing alternative serological methods for diagnosis of *Leptospira* infections.

We have previously reported the development of enzyme immunoassays capable of detecting bovine antibodies to specific individual *Leptospira* serovars (19–22). We report here, the development and evaluation of an indirect ELISA which is capable of detecting bovine antibodies to multiple pathogenic *Leptospira* serovars. This immunoassay uses an antigen composed of a mixture of killed whole cells of the 6 serovars that are routinely monitored in Canada. Although protein antigens which cross-react among *Leptospira* serovars have been identified (23–26), we chose to use sonicated whole cells as the antigen in this assay. This is primarily because the immunodominant antigens of *Leptospira* are the serovar-specific lipopolysaccharide (LPS) components (27–29) and not the cross-reactive proteins. Thus, the use of an antigen which is comprised of a mixture of whole cells from several serovars should enable the assay to detect, not only the antibodies against the proteins that cross-react among the serovars, but also the antibodies to the immunodominant LPS components which are specific for each individual serovar. Depending on the geographic location in which the test is to be used, the composition of the antigen can be varied to include cells from the serovars prevalent in that region.

The relative sensitivity and specificity estimates of this ELISA were approximately 94% and 95%, respectively, at the recommended cut-off point of $\geq$ 42% HPC. Of the 39 MAT-positive sera which were ELISA-negative at this cut-off point, 31 reacted in the MAT to a single serovar and the others to 2 or more serovars. In these 39 sera, the MAT detected antibodies to serovars *pomona*, *hardjobovis*, *copenhageni*, *canicola*, and *sejroe*. The MAT titres of these sera were predominantly 100, but some were as high as 400. It is possible that the anti-*Leptospira* antibodies in these sera were of an isotype not recognized by the anti-bovine IgG_1 antibody that was used to construct the ELISA conjugate. This needs further investigation. It is also possible that some of these sera may have been falsely positive in the MAT. The 165 MAT-negative sera which were scored positive in the ELISA may have contained non-agglutinating anti-*Leptospira* antibodies which are not detectable by the MAT. It is also possible that some of these sera may have been falsely positive in the ELISA. If a higher sensitivity (or specificity) than that obtained at the recommended cut-off point is desired for this ELISA, this can be accomplished by the appropriate adjustment of the cut-off point as demonstrated in Table I.

This assay is capable of detecting antibodies in the sera of cows experimentally infected with *hardjobovis*, as early as 1 wk postinoculation. Antibodies were detected in the sera of cows similarly infected with serovars *canicola*, *copenhageni*, and *grippotyphosa* at 2 to 4 wk postinoculation. The differences in the times at which the ELISA detected the antibodies to the various serovars is likely not attributable to the performance of the assay, but instead may be due to the nature of the organisms that were used for infection. A field strain of *hardjobovis* which had undergone relatively few passages was used for this experiment. In contrast, the other 3 serovars were strains that had been passaged on a weekly basis for many years. The cows inoculated with the *hardjobovis* organisms probably became infected and developed an immune response more quickly compared to those animals inoculated with each of the other 3 serovars. The MAT results obtained with these sera generally support this assertion.

This ELISA offers many advantages over the MAT. It uses non-hazardous reagents, it is relatively sensitive and specific and it is semi-automated. Furthermore, the rigorous quality control criteria that have been established for this assay ensure the reproducibility of results, which are interpreted objectively. The use of frozen antigen-coated plates provides opportunity for testing 5 d per week. Potentially, this ELISA can be used as a screening test for leptospirosis. Sera that are scored positive in this screening ELISA can then be tested with serovar-specific ELISAs (if available), or with the MAT, to determine the identity of the serovar that caused the infection.

In conclusion, we report the development and evaluation of a sensitive and specific ELISA for detection of bovine antibodies to multiple pathogenic *Leptospira* serovars. This assay shows good potential for use as a screening assay for bovine leptospirosis.

Acknowledgments

The authors thank Mr. S. Duff for assistance with the MAT and Dr. C. Bolin for providing us with the *hardjobovis* field strain. We also thank the staff of the CFIA regional laboratories for collecting the bovine field sera.

References

1. Faine S. *Leptospira* and Leptospirosis. Boca Raton: CRC Press, 1994:117–144.
2. Thiermann AB. Leptospirosis: Current developments and trends. J Am Vet Med Assoc 1984;184:722–725.
3. Robertson A, Boulanger P, Mitchell D. Isolation and identification of a leptospire of the hebdomadis serogroup (*L. hardjo*) from cattle in Canada. Can J Comp Med 1964;28:13–18.
4. Andress CE, Greenfield J, Macdonald K. Some *Leptospira* agglutinins detected in domestic animals in British Columbia. Can J Comp Med 1976;40:215–217.
5. Higgins R, Cayouette P, Hoquet F, De LaSalle F. Serological studies on leptospirosis in domestic animals in Quebec. Can J Comp Med 1980;44:229–231.
6. Kingscote BF. Leptospirosis in livestock. Can Vet J 1985;26: 235–236.
7. Kingscote BF. The diagnosis of *Leptospira* serovar *hardjo* infection in cattle in Canada. Can Vet J 1985;26:270–274.
8. Prescott JF, Miller RB, Nicholson VM. Isolation of *Leptospira hardjo* from kidneys of Ontario cattle at slaughter. Can J Vet Res 1987;51:229–231.

9. Prescott JF, Miller RB, Nicholson VM, Martin SW, Lesnick T. Seroprevalence and association with abortion of leptospirosis in cattle in Ontario. Can J Vet Res 1988;52:210–215.
10. Richardson GF, Spangler E, MacAulay EB. A serological survey of 4 *Leptospira* serovars in dairy cows on Prince Edward Island. Can Vet J 1995;36:769–770.
11. Cole JR, Sulzer CR, Pursell AR. Improved microtechnique for the leptospiral microscopic agglutination test. Appl Microbiol 1973;25:976–980.
12. Canadian Council on Animal Care. Guide to the Care and Use of Experimental Animals. Olfert ED, Cross BM, McWilliam AA, eds. Vol 1, 2nd ed. Ottawa: Canadian Council on Animal Care, 1993.
13. Ellis WA, O'Brien JJ, Neill SD, Hanna, J. Bovine leptospirosis: Serological findings in aborting cows. Vet Rec 1982;110: 178–180.
14. White FH, Sulzer KR, Engel RW. Isolations of *Leptospira interrogans* serovars *hardjo, balcanica* and *pomona* from cattle at slaughter. Am J Vet Res 1982;43:1172–1173.
15. Thiermann AB. Bovine leptospirosis: Bacteriologic versus serologic diagnosis of cows at slaughter. Am J Vet Res 1983;44: 2244–2245.
16. Ellis WA, McParland PJ, Bryson DG, Cassels JA. Boars as carriers of leptospires of the Australis serogroup on farms with an abortion problem. Vet Rec 1986;118:563.
17. Ellis WA, McParland PJ, Bryson DG, Thiermann AB, Montgomery J. Isolations of leptospires from the genital tract and kidneys of aborted sows. Vet Rec 1986;118:294–295.
18. Ellis WA, Thiermann AB. Isolation of leptospires from the genital tract of Iowa cows. Am J Vet Res 1986;47:1694–1696.
19. Surujballi O. An indirect enzyme-linked immunosorbent assay for the detection of *Leptospira interrogans* serovar *pomona* antibodies in bovine sera. J Rapid Methods Automation Microbiol 1997;5:179–191.
20. Surujballi O, Henning D, Marenger R, Howlett C. Development of a monoclonal antibody-based competitive enzyme-linked immunosorbent assay for the detection of *Leptospira borgpetersenii* serovar *hardjo* type hardjobovis antibodies in bovine sera. Can J Vet Res 1997;61:267–274.
21. Surujballi OP, Marenger RM, Eaglesome MD, Sugden EA. Development and initial evaluation of an indirect enzyme-linked immunosorbent assay for the detection of *Leptospira interrogans* serovar *hardjo* antibodies in bovine sera. Can J Vet Res 1997;61: 260–266.
22. Surujballi O, Mallory M. Competitive enzyme-linked immunosorbent assay for detection of *Leptospira interrogans* serovar *pomona* antibodies in bovine sera. Clin Diagn Lab Immunol 2001;8:40–43.
23. Jost BH, Adler B, Faine S. Reaction of monoclonal antibodies with species specific determinants in *Leptospira interrogans* outer envelope. J Med Microbiol 1988;27:51–57.
24. Mendoza L, Prescott JF. Serodiagnosis of leptospirosis in pigs using an axial filament enzyme-linked immunosorbent assay. Vet Microbiol 1992;31:55–70.
25. Nicholson VM, Prescott JF. Outer membrane proteins of three pathogenic *Leptospira* species. Vet Microbiol 1993;36:123–138.
26. Lin M, Surujballi O, Nielsen K, Nadin-Davis S, Randall G. Identification of a 35-kilodalton serovar-cross-reactive flagellar protein, FlaB, from *Leptospira interrogans* by N-terminal sequencing, gene cloning, and sequence analysis. Infect Immun 1997;65: 4355–4359.
27. Tsuji M, Kawaoka Y, Naiki M, Yanagawa R. Isolation of antigenically active components from leptospiral serovar-specific lipopolysaccharide antigen by alkaline treatment. Microbiol Immunol 1981;25:949–957.
28. Ono E, Takase H, Naiki M, Yanagawa R. Purification, characterization and serological properties of a glycolipid antigen reactive with a serovar-specific monoclonal antibody against *Leptospira interrogans* serovar *canicola.* J Gen Microbiol 1987;133:1329–1336.
29. Cinco M, Banfi E, Panfili E. Leptospiral lipopolysaccharide presence in the outer envelope: Electrophoretic evidence and immunological specificity. Zentralbl Bakteriol Hyg A 1988;269: 277–283.